

North American West Nile virus genotype isolates demonstrate differential replicative capacities in response to temperature

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The presence of West Nile virus (WNV) was first documented in California, USA, during the summer of 2003, and subsequently the virus has become endemic throughout the state. Sequence analysis has demonstrated that the circulating strains are representative of the North American (WN02) genotype that has displaced the East Coast genotype (NY99). A recent study has indicated that enhanced vector competence at elevated temperatures may have played a role in the displacement of the East Coast genotype by WN02. In the current study, four WN02 strains from California, including an initial 2003 isolate (COAV997), were compared to strain NY99 in growth curve assays in mosquito and duck embryonic fibroblast (DEF) cell lines at differing, biologically relevant temperatures to assess the relative temperature sensitivities of these natural isolates. COAV997 was significantly debilitated in viral replication in DEF cells at 44 °C. Full-length sequence comparison of COAV997 against the NY99 reference strain revealed non-synonymous mutations in the envelope glycoprotein (V159A), non-structural protein 1 (NS1) (K110N) and non-structural protein 4A (NS4A) (F92L), as well as two mutations in the 3' UTR: C→T at nt 10 772 and A→G at nt 10 851. These non-synonymous mutations were introduced into the NY99 viral backbone by site-directed mutagenesis. A mutant containing the NS1-K110N and NS4A-F92L mutations exhibited a debilitated growth phenotype in DEF cells at 44 °C, similar to that of COAV997. One explanation for the subsistence of this genotype is that COAV997 was obtained from an area of California where avian host species might not present elevated temperatures. These data indicate that the NS1 and NS4A mutations identified in some WN02 isolates could reduce thermal stability and impede replication of virus at temperatures observed in febrile avian hosts.

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INTRODUCTION

West Nile virus (WNV; family *Flaviviridae* genus *Flavivirus*) is maintained and amplified in a two-host transmission cycle between *Culex* spp. mosquitoes and multiple bird species. When replicating in this two-host system, the virus is exposed to very different host environments, including disparate immune systems, host-cell receptors and temperatures, all of which can affect viral replication. The low genetic substitution rates described in flaviviruses, such as WNV, are generally attributed to the strong purifying selective effect of replication in the presence of such disparate factors (Coffey *et al.*, 2008;

Jerzak *et al.*, 2008; Scott *et al.*, 1994). Temperature plays an important role in viral replication rates and transmission, affecting the length of the extrinsic incubation, the seasonal phenology of mosquito host populations and geographical variation in human case incidence (Kilpatrick *et al.*, 2008; Kinney *et al.*, 2006; Reisen *et al.*, 2006). West Nile virus is capable of replication across a wide range of temperatures, from as low as 14 °C in poikilothermic mosquitoes (Cornel *et al.*, 1993; Reisen *et al.*, 2006) to 45 °C in febrile avian hosts (Kinney *et al.*, 2006). For transmission to occur, the virus must maintain a eurythermic replicative capacity, and retention of this thermal plasticity may act as an evolutionary constraint.

West Nile virus was first identified in North America in 1999 (Lanciotti *et al.*, 1999), and the founding strain (NY99) was representative of the East Coast genotype

The GenBank/EMBL/DDBJ accession numbers for the sequences of the WNV isolates reported in this paper are JF703161, JF703162, JF703163 and JF703164.

(Lanciotti *et al.*, 2002). NY99 is highly virulent for birds in the family Corvidae (Brault *et al.*, 2007; Eidson *et al.*, 2001; Wheeler *et al.*, 2009) and has caused extensive morbidity and mortality in humans and equines (Hayes *et al.*, 2005). Concurrent with the westward expansion of WNV, a new North American genotype (WN02) emerged, and by 2004 had largely displaced the East coast genotype in the United States (Davis *et al.*, 2005). The WN02 genotype has been typified by the presence of a conserved valine-to-alanine mutation in the envelope glycoprotein (E) at amino acid 159 (E-V159A; Davis *et al.*, 2005). The first isolation of WNV in California, USA, was made from a pool of *Culex tarsalis* collected during July 2003 in Imperial County, a desert biome adjacent to the Mexican border (Reisen *et al.*, 2004). Strains associated with this initial introduction of WNV to California were found to contain the E-V159A mutation and phylogenetically clustered with other WN02 genotype viruses (Davis *et al.*, 2005; Deardorff *et al.*, 2006). Transmission resumed in 2004 with the northward spread of WNV into central and northern California, with a notably high incidence of morbidity and mortality in corvid populations (Hom *et al.*, 2005), such as the yellow-billed magpie (*Pica nuttalli*) (Ernest *et al.*, 2010).

In an effort to determine what phenotypic advantages facilitated WN02 displacement of the East Coast genotype, several *in vitro* and *in vivo* studies have compared isolates of the two genotypes (Ebel *et al.*, 2004; Kilpatrick *et al.*, 2008; Moudy *et al.*, 2007). Kilpatrick *et al.* (2008) reported that warmer temperature disproportionately increased replication of WN02 virus, compared with NY99, in *Culex* spp. mosquitoes, thereby allowing WN02 to be transmitted more rapidly after infection, leading to eventual competitive displacement of NY99. The current study addressed the hypothesis that WN02 strains have a greater eurythermic capacity by comparing the replicative capacities of California WN02 strains with the founding North American NY99 genotype strain at temperature extremes in mosquito and avian cell lines. The strains utilized in these experiments vary by locality, year and type of host. Previous studies comparing WN02 and NY99 have been conducted either *in vitro* or *in vivo* at a single temperature

(Ebel *et al.*, 2004; Moudy *et al.*, 2007), with one *in vivo* study investigating the effect of temperatures up to 32 °C on WNV infection and transmission by mosquitoes (Kilpatrick *et al.*, 2008). The current study assessed WNV replication over a wider range of biologically relevant temperatures within vertebrate and invertebrate systems to assess the potential effect of host selection for restricting expansion of viruses with high fitness under specific temperature ranges. The *Aedes albopictus*-derived cell line (C6/36) was maintained at 22, 28 and 34 °C to simulate ambient temperatures during the spring/early summer, summer and late summer/early autumn in California. Duck embryonic fibroblasts (DEF) were maintained at 37, 41 and 44 °C to simulate the low, average and high avian body temperatures observed during terminal infection (Kinney *et al.*, 2006). Mutations associated with altered replicative capacities at different temperatures were implicated by introducing them into an East Coast genotype, NY99, infectious cDNA clone by site-directed mutagenesis. These studies identified genetic modifications that are potentially associated with different replicative capacities at temperature extremes that could have mediated the evolution of WNV as it became established throughout California.

RESULTS

Sequence analysis of the natural isolates

Full-length consensus sequences obtained from the natural isolates COAV997, IMP116, COAV689 and COAV2900 (Table 1) were analysed by comparing them with the NY99 reference genotype strain (Table 2). All four isolates contained non-synonymous mutations at genomic nucleotide position 1442 that associated with the predicted amino acid substitution of E-V159A, as well as the 3' UTR A/G→G substitution at nt 10 851 (3'UTR-a/g10851g). The full-length sequence obtained from COAV997 revealed additional non-synonymous mutations that resulted in predicted amino acid substitutions in non-structural proteins 1 (NS1) (K110N) and 4A (NS4A) (F92L) (Table 2), as

Table 1. Origin and passage history of California natural isolates

GenBank accession no.	Strain	Location*	Year	Host	Passage history
AF196835	382-99 (IC) (NY99-IC)	New York	1999	<i>Phoenicopterus chilensis</i>	Vero-1 (BHK-transfection)
JF703162	CA-03 COAV997 (COAV997)	Imperial Valley	2003	<i>Cx. tarsalis</i>	Vero-1
DQ080055	CA-03 IMPR 102 (IMP102)	Imperial Valley	2003	<i>Cx. tarsalis</i>	Vero-1
JF703164	CA-03 IMPR 116 (IMP116)	Imperial Valley	2003	<i>Cx. tarsalis</i>	Vero-1
DQ080056	CA-03 IMPR 1075 (IMP1075)	Imperial Valley	2003	<i>Cx. tarsalis</i>	Vero-1
DQ080059	CA-04 04-7168 (CA-04)	Sacramento County	2004	<i>P. nuttalli</i>	Vero-4
JF703161	CA-04 COAV689 (COAV689)	Coachella Valley	2004	<i>Cx. tarsalis</i>	Vero-1
JF703163	CA-05 COAV2900 (COAV2900)	Coachella Valley	2005	<i>Cx. tarsalis</i>	Vero-1

*All locations are in California, USA, with the exception of New York, USA.

Table 2. Comparison of non-synonymous and non-coding mutations of California natural isolates as compared to strain NY99

Virus strains in bold were sequenced in the current study, while others were published previously [NY99 strain 382-99 (Lanciotti *et al.*, 1999) and strains IMP102, IMP1075 and CA-04 (Deardorff *et al.*, 2006)]. Italics indicate the nucleotide or amino acid substitutions in the COAV997 strain that were introduced into the NY99 virus backbone by site-directed mutagenesis. Mutations marked with lower case letters indicate a change in nucleotide, upper case letters indicate a change in amino acid.

Nucleotide position	Amino acid position	Virus strain							
		NY99	COAV997	IMP102	IMP116	IMP1075	CA-04	COAV689	COAV2900
19	5' UTR	g						a	
458	C-121	V							A
1442	E-159	V	A	A	A	A	A	A	A
2799	NS1-110	K	N	N	N	N		N	
6263	NS3-551	V				A			
6721	NS4A-85	A							T
6742	NS4A-92	F	L		L/F	L		L	
6871	NS4A-135	V				M		M	
6901	NS4A-145	S					G		
6992	NS4B-26	N							S
8620–21	NS5-314	K							R
10 213	NS5-845	G			R				
10 279	NS5-867	N							D
10 384	NS5-902	D							N
10 403	3' UTR	t							c
10 435	3' UTR	c							t
10 459	3' UTR	t							c
10 682	3' UTR	g							a
10 772	3' UTR	c	t	t	t	t		t	
10 851	3' UTR	a/g	g	g	g	g	g	g	g
10 980	3' UTR	c		a					
11 009	3' UTR	t						c	

well as a nucleotide substitution in the 3' UTR [C→T at nt 10 772 (3'UTR-c10772t)]. The IMP116 isolate exhibited predicted amino acid substitutions at NS1-K110N, NS4A-F92L/F and NS5-G845R (Table 2). The full-length sequence of the COAV689 isolate revealed amino acid substitutions at NS1-K110N, NS4A-F92L and NS4A-V135M, as well as nucleotide substitutions in the 3' UTR [3'UTR-c10772t and T→C at nt 11 009 (3'UTR-t11009c); Table 2], whereas analysis of the COAV2900 isolate demonstrated amino acid substitutions at capsid (C)-V121A, NS4A-A85T, NS4B-N26S, NS5-K314R, NS5-N867D and NS5-D902N, as well as multiple nucleotide substitutions in the 3' UTR (T→C at nt 10 403, C→T at nt 10 435, T→C at nt 10 459 and G→A at nt 10 682; Table 2).

Mosquito cell (C6/36) temperature-specific growth profiles of California WNV strains

Viral growth was assessed in C6/36 cells at 22, 28 and 34 °C (Fig. 1) for a subset of the California strains (COAV997, IMP102, CA-04 and COAV2900) and infectious-clone-derived NY99 (NY99-IC) viruses (Table 1). COAV997 was the initial isolate of WNV from California and therefore was selected initially to represent the founding genotype. An infectious-clone-derived COAV997 strain (COAV997-IC)

was also generated for use as a California reference strain and was utilized for comparisons with the corresponding natural COAV997 isolate. A repeated measures analysis of variance (ANOVA) with viral strains and temperatures as main effects and days after inoculation as the repeated measure indicated that there was no significant difference in the interaction between virus strains and temperature ($P=0.999$). The ranges of the overall mean titres at each temperature were as follows: 4.5–5.1 log₁₀ p.f.u. ml⁻¹ at 22 °C, 6.9–7.1 log₁₀ p.f.u. ml⁻¹ at 28 °C and 7.4–7.9 log₁₀ p.f.u. ml⁻¹ at 34 °C.

Avian cell (DEF) temperature-specific growth profiles of California WNV strains

A repeated measures ANOVA was used to compare viral growth profiles of NY99-IC, COAV997, COAV997-IC, IMP102, CA-04 and COAV2900 in DEF cells at 37, 41 and 44 °C (Fig. 2). There was a significant interaction between virus strain and temperature ($P<0.001$), and most of the variance could be ascribed to temperature and time ($P<0.001$). *Post-hoc* analysis of the datasets for each individual temperature revealed that the overall mean titres for COAV997 and COAV997-IC were in a unique group that did not contain IMP102, CA-04, COAV2900

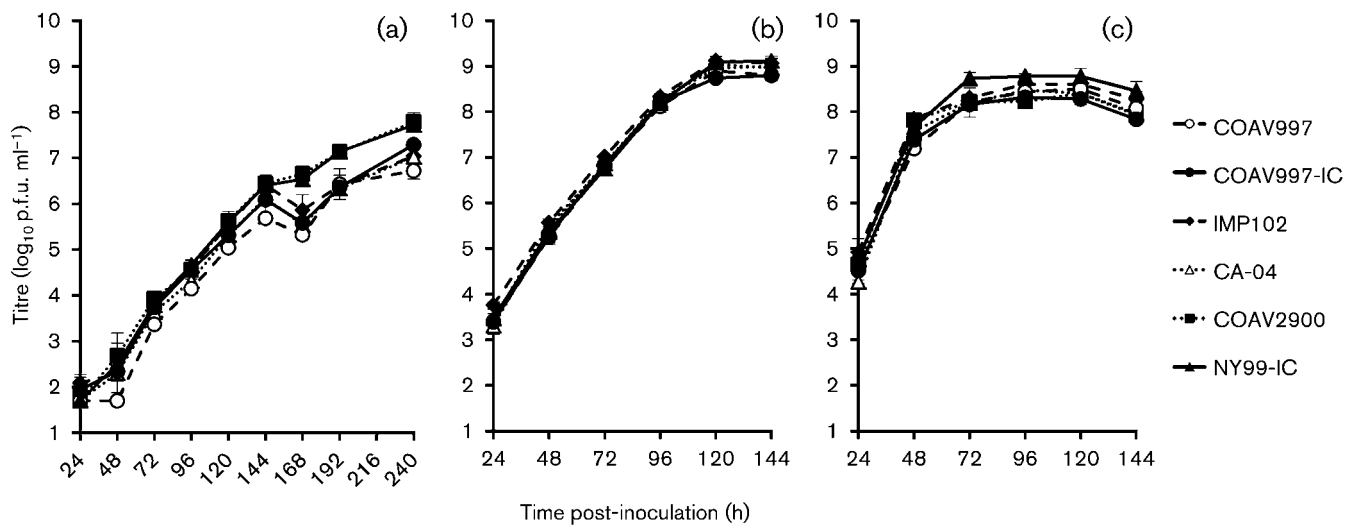


Fig. 1. Growth curves of natural California isolates in C6/36 cells at (a) 22, (b) 28 and (c) 34 °C. Error bars represent SD and the limit of detection is 1.7 log₁₀ p.f.u. ml⁻¹.

and NY99-IC at 37 °C or 44 °C (Tukey–Kramer multiple comparisons, $\alpha=0.05$), with overall mean titres correspondingly ranging from 7.5–7.9 and 1.7–6.0 log₁₀ p.f.u. ml⁻¹ at 37 and 44 °C, respectively (Table 3). No significant differences were found at 41 °C by this pairwise comparison of the overall means (Table 3); however, COAV997 and COAV997-IC peaked on day 3 post-inoculation (p.i.), while the other strains peaked on day 2 p.i. (Fig. 2b). Peak titres at 44 °C occurred on day 3 p.i. for IMP102, CA-04, COAV2900 and NY99-IC. Both COAV997 and COAV997-IC were debilitated at 44 °C, with a detectable virus titre (>1.7 log₁₀ p.f.u. ml⁻¹) being observed in none of the

COAV997 replicates and in only one of three replicates for COAV997-IC. The peak titre for that replicate was 4.4 log₁₀ p.f.u. ml⁻¹ on day 5 p.i. As virus was detected in only one flask of the infectious-clone-derived virus and not in the COAV997 natural isolate, plaques were analysed to determine whether any compensatory mutations were generated in this single replicate. Full-length consensus sequencing of an individual plaque showed no additional mutations. Notably, since the COAV997-IC was engineered by site-directed mutagenesis using the two-plasmid system of NY99 as a template, it therefore contained additional non-synonymous mutations, C→T

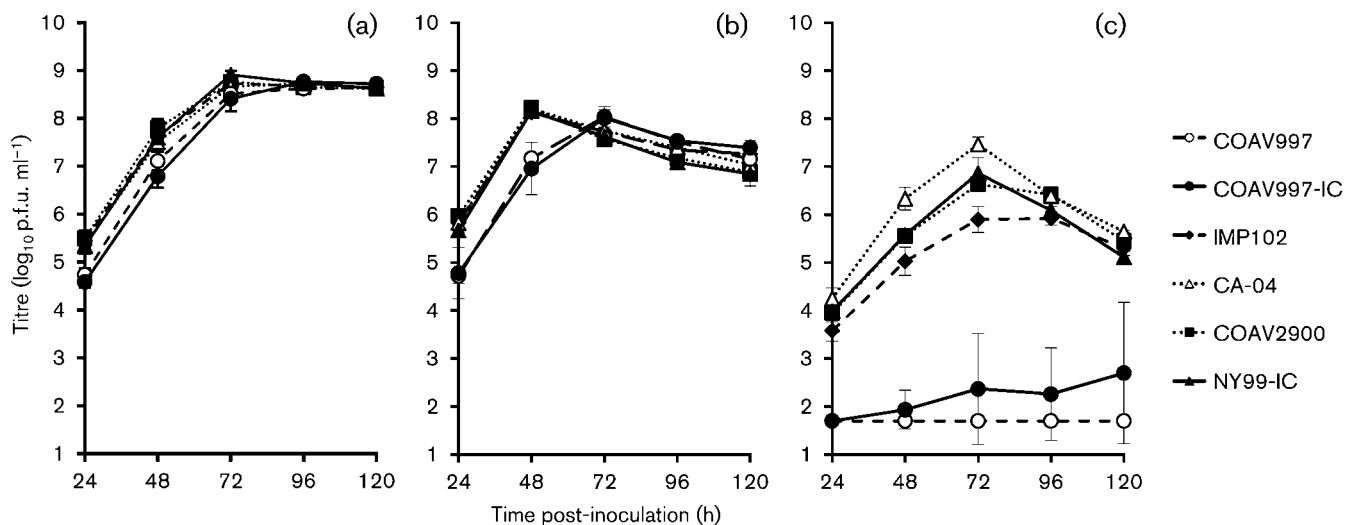


Fig. 2. Growth curves of natural California isolates in DEF cells at (a) 37, (b) 41 and (c) 44 °C. Error bars represent the SD and the limit of detection is 1.7 log₁₀ p.f.u. ml⁻¹.

Table 3. Summary of overall mean titres in DEF cells at 37, 41 and 44 °C

Horizontal lines indicate the three groups of data from independent experiments.

Virus strain	Overall mean virus titre (p.f.u. ml ⁻¹)					
	37 °C*	41 °C*	44 °C*	Δ ₃₇₋₄₁ †	Δ ₄₁₋₄₄ †	Δ ₃₇₋₄₄ †
COAV997	7.5 ^a	6.9 ^a	1.7 ^a	0.6	5.2	5.8
COAV997-IC	7.5 ^a	6.9 ^a	2.2 ^a	0.6	4.7	5.3
IMP102	7.8 ^b	7.3 ^a	5.1 ^b	0.5	2.2	2.7
CA-04	7.8 ^b	7.2 ^a	6.0 ^b	0.6	1.2	1.8
COAV2900	7.9 ^b	7.2 ^a	5.6 ^b	0.7	1.6	2.3
NY99	7.8 ^b	7.1 ^a	5.5 ^b	0.7	1.6	2.3
COAV997	7.7 ^{ab}	7.5 ^a	1.9 ^a	0.2	5.6	5.8
COAV997-IC	7.5 ^a	7.3 ^a	2.9 ^b	0.2	4.4	4.6
E	7.6 ^a	7.5 ^a	6.0 ^c	0.1	1.5	1.6
NS1	7.4 ^a	7.4 ^a	5.5 ^c	0	1.9	1.9
E.NS1	7.5 ^a	7.5 ^a	5.7 ^c	0	1.8	1.8
3' UTR	8.0 ^b	7.6 ^a	5.9 ^c	0.4	1.7	2.1
NY99	7.9 ^b	7.4 ^a	6.0 ^c	0.5	1.4	1.9
COAV997	8.1 ^a	7.4 ^b	1.7 ^a	0.7	5.7	6.4
COAV997-IC	8.0 ^a	7.2 ^{ab}	2.6 ^a	0.8	4.6	5.4
NS1	7.8 ^a	7.1 ^a	5.9 ^c	0.7	1.2	1.9
NS4A	8.1 ^a	7.0 ^a	5.4 ^{bc}	1.1	1.6	2.7
NS1.NS4A	7.9 ^a	7.0 ^a	1.9 ^a	0.9	5.1	6.0
E.NS1.3'UTR	8.0 ^a	7.4 ^b	4.3 ^b	0.6	3.1	3.7
NY99	8.1 ^a	7.2 ^{ab}	6.3 ^c	0.9	0.9	1.8

*Means followed by the same superscripts (a, b and c) were not significantly different by pairwise analysis (Tukey–Kramer multiple comparisons test, $\alpha < 0.05$).

†The difference in overall mean titre between 37–41 °C, 41–44 °C and 37–44 °C, as indicated.

at nt 3880 (NS2A-H118Y) and A→G at nt 4922 (NS3-K104R) (Beasley *et al.*, 2005; Kinney *et al.*, 2006), that were present within the NY99 sequence. These additional non-synonymous mutations may have contributed to the slight replicative capacity of COAV997-IC at 44 °C.

Avian cell (DEF) temperature-specific growth profiles of engineered WNV mutants

Non-synonymous and non-coding mutations observed in COAV997 (relative to the NY99 reference strain) were introduced into the NY99 viral backbone by site-directed mutagenesis in order to assess the phenotypic contribution of specific mutations either individually or in combination. The following mutants were engineered and utilized in growth curve assessments: (i) E-V159A (E), (ii) NS1-K110N (NS1), (iii) NS4A-F92L (NS4A), (iv) 3' UTR-c10772t (3'UTR), (v) E-V159A with NS1-K110N (E.NS1), (vi) NS1-K110N with NS4A-F92L (NS1.NS4A), and (vii) E-V159A with NS1-K110N and 3'UTR-c10772t (E.NS1.3'UTR).

The first of two separate experiments utilized the E, NS1, E.NS1 and 3'UTR mutants with NY99-IC, COAV997 and COAV997-IC as controls (Fig. 3). A repeated measures ANOVA was used to compare growth profiles of these

viruses in DEF cells at 37, 41 and 44 °C; a significant interaction between virus strain and temperature was observed, with most of the variance ascribed to temperature and time ($P < 0.001$). Therefore, *post-hoc* analyses were performed on the data for each individual temperature. Pairwise comparisons of the overall means for each temperature resulted in overlapping groups at 37 °C and no differences at 41 °C, but significant differences were observed at 44 °C, with mean titres ranging from 1.9 to 6.0 log₁₀ p.f.u. ml⁻¹ (Table 3, Tukey–Kramer multiple comparisons, $\alpha = 0.05$). The peak titre at 44 °C occurred on day 3 p.i. for NY99-IC and the 3'UTR mutant, while the E, NS1 and E.NS1 mutants peaked at day 4 p.i. (Fig. 3c). Detectable titres were found in all three replicates of the COAV997-IC in DEF at 44 °C, and peak titre occurred at day 4 p.i. COAV997 was debilitated in viral replication at 44 °C, with virus detected in one of three replicates on day 1 p.i. (2.0 log₁₀ p.f.u. ml⁻¹); a second replicate demonstrated detectable virus peaking on day 4 p.i. (2.7 log₁₀ p.f.u. ml⁻¹).

The second experiment utilized the NS1, NS4A, NS1.NS4A and E.NS1.3'UTR mutants with NY99-IC, COAV997 and COAV997-IC as controls (Fig. 4). A repeated measures ANOVA compared growth profiles of these viruses in DEF cells at 37, 41 and 44 °C, and again there was a significant

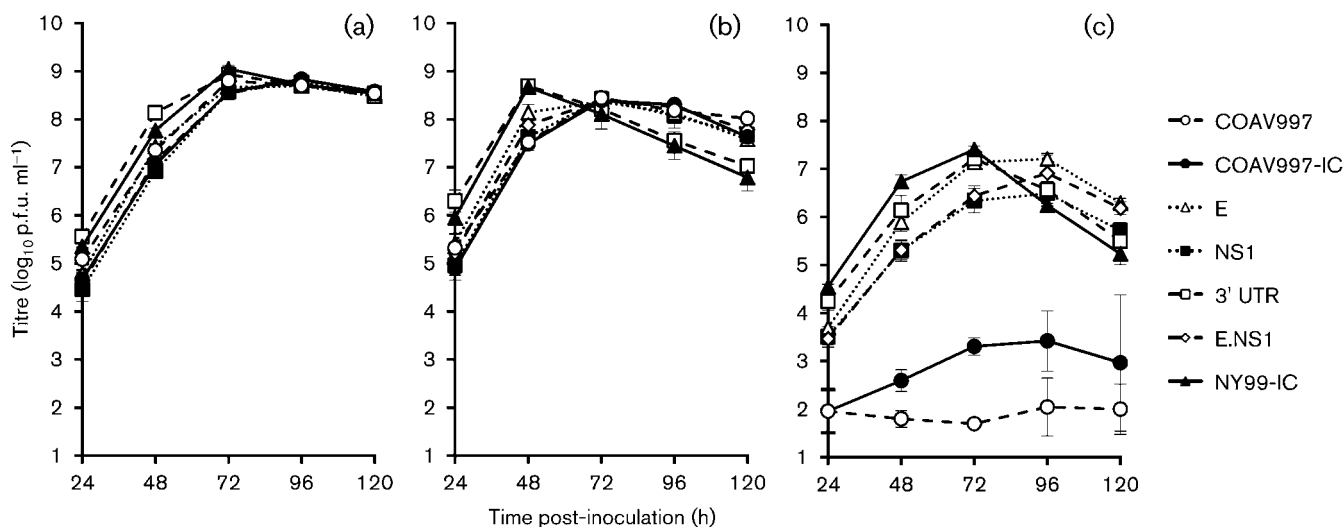


Fig. 3. Growth curves of engineered mutants in DEF at (a) 37, (b) 41 and (c) 44 °C. Error bars represent SD and the limit of detection is 1.7 log₁₀ p.f.u. ml⁻¹.

interaction between virus strain and temperature, with most of the variance being ascribed to temperature and time ($P < 0.001$). Therefore, a *post-hoc* analysis was performed on the data for each temperature. Pairwise comparison of the overall means resulted in no significant differences in DEF at 37 or 41 °C; however, significant differences were observed at 44 °C, with mean titres ranging from 1.7–6.3 log₁₀ p.f.u. ml⁻¹ (Table 3; Tukey–Kramer multiple comparisons, $\alpha = 0.05$). At 44 °C, the NY99-IC strain elicited a peak viral titre on day 3 p.i. and detectable titre was found in two of the three replicates of the COAV997-IC strain, with the average occurring on day 5 p.i. COAV997 was debilitated in viral replication at 44 °C, with a detectable titre occurring in only

one replicate on day 1 p.i. (2.2 log₁₀ p.f.u. ml⁻¹). The NS1 and NS4A individual mutants both peaked on day 4 p.i. at 44 °C, whereas the E.NS1.3'UTR mutant did not peak within 5 days p.i. (Fig. 4c). Detectable titres were found in two of the three replicates of the NS1.NS4A mutant, with peak titres of 2.7 log₁₀ p.f.u. ml⁻¹ on day 1 p.i. in one flask and 2.4 log₁₀ p.f.u. ml⁻¹ on day 5 p.i. in the other. Analysis of the overall mean titres for NS1.NS4A, COAV997 and COAV997-IC revealed that these viruses were not significantly different at 44 °C (Table 3), implicating the NS1-K110N and NS4A-F92L mutations as genetic determinants of the highly temperature sensitive (*ts*) phenotype observed for the COAV997 strain.

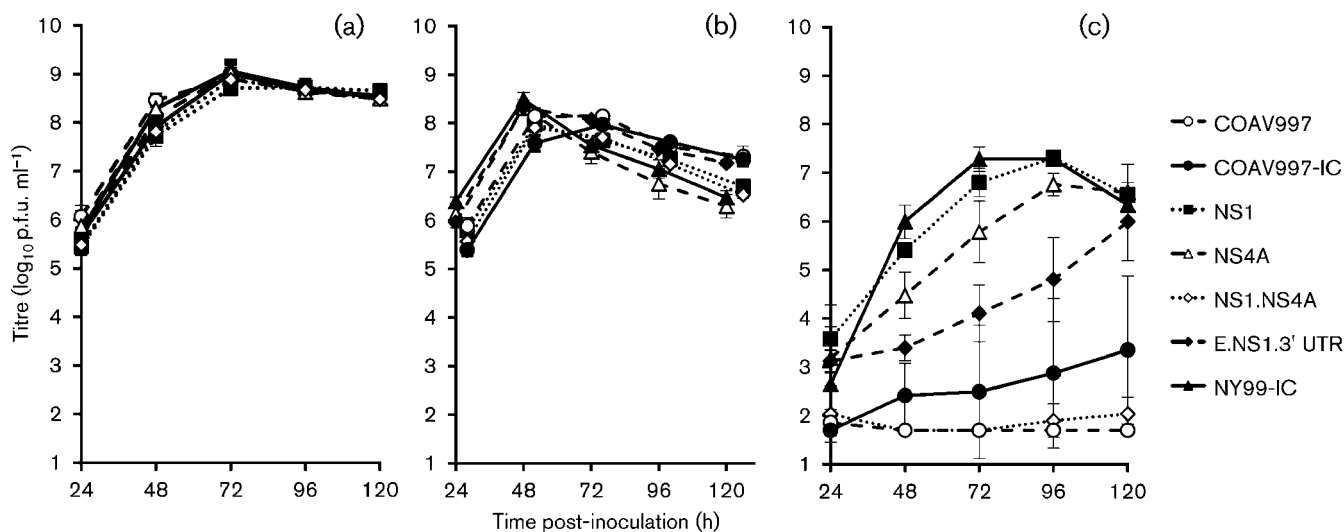


Fig. 4. Growth curves of additional engineered mutants in DEF at (a) 37, (b) 41 and (c) 44 °C. Error bars represent SD and the limit of detection is 1.7 log₁₀ p.f.u. ml⁻¹. Data points on Fig. 4b have been slightly offset for presentation purposes.

Comparative Vero cell plaque morphologies

Plaque morphologies were described for a subset of the California strains (COAV997, IMP102, CA-04 and COAV2900), as well as NY99-IC and the NS1.NS4A mutant, on Vero cells at 37 and 41 °C (Fig. 5). A comparison could not be made in Vero cells at 44 °C owing to the intolerance of high temperature by these mammalian cells. At 37 °C, the viruses all exhibited similar plaque phenotypes (not shown); however, when compared at 41 °C, differences in plaque size became apparent. The viruses assessed exhibited the following mean plaque sizes \pm SD: COAV997, 1.5 ± 0.6 mm; IMP102, 2.7 ± 0.4 mm; CA-04, 3.4 ± 0.3 mm; COAV2900, 3.0 ± 0.3 mm; NY99-IC, 3.1 ± 0.3 mm; and NS1.NS4A, 1.8 ± 0.7 mm. The mean plaque size for COAV997 and NS1.NS4A were not significantly different from each other; however, they were significantly different than NY99-IC, IMP-102, CA-04 and COAV2900 (Tukey's multiple comparison test, $P < 0.05$). These data lend support to the contention that of the NS1-K110N and NS4A-F92L mutations contribute to the *ts* phenotype of the COAV997 isolate.

DISCUSSION

Previous studies have demonstrated a more rapid dissemination of the WN02 strain in *Culex* spp. mosquitoes held at elevated temperatures when compared with the NY99 North American founding genotype (Kilpatrick *et al.*, 2008), thus suggesting a selective role for temperature in the adaptive landscape of WNV. In the current study, West

Coast representatives of the WN02 genotype were compared to NY99 at a range of biologically relevant mosquito and avian temperatures, and, although most strains assayed were tolerant to replication at the extreme, high temperature (44 °C), one strain, COAV997, was found to be non-permissive for replication at this temperature. Comparison of growth profiles for these strains in the C6/36 mosquito cell line at lower temperatures revealed no statistical differences (Fig. 1). COAV997 and NY99-IC were previously assayed in a *Cx. tarsalis*-derived cell line (*CxtarD1*), which also resulted in no significant virus versus temperature interaction (Andrade *et al.*, 2010). The COAV997 strain originated from a *Cx. tarsalis* mosquito pool collected in Imperial County, California, USA, during July 2003. IMP102, the first positive pool recorded in California, was obtained at nearly the same time (July 2003) and in close proximity (within 40 km) of COAV997, yet IMP102 did not show the severe *ts* effect observed with COAV997, indicating that California was invaded by multiple strains of WNV. Full-length sequence comparison of these two strains revealed that they both contained NS1-K110N and 3'UTR-c10772t, and only differed by two non-synonymous mutations, NS4A-F92L and a C→A change in the 3' UTR at nt 10 980 (Table 2).

To determine which mutations contributed to the debilitated replicative phenotype in DEF at 44 °C, point mutants were engineered into the NY99 backbone by using site-directed mutagenesis, and growth curves were analysed for the various temperatures. The overall means of E-V159A,

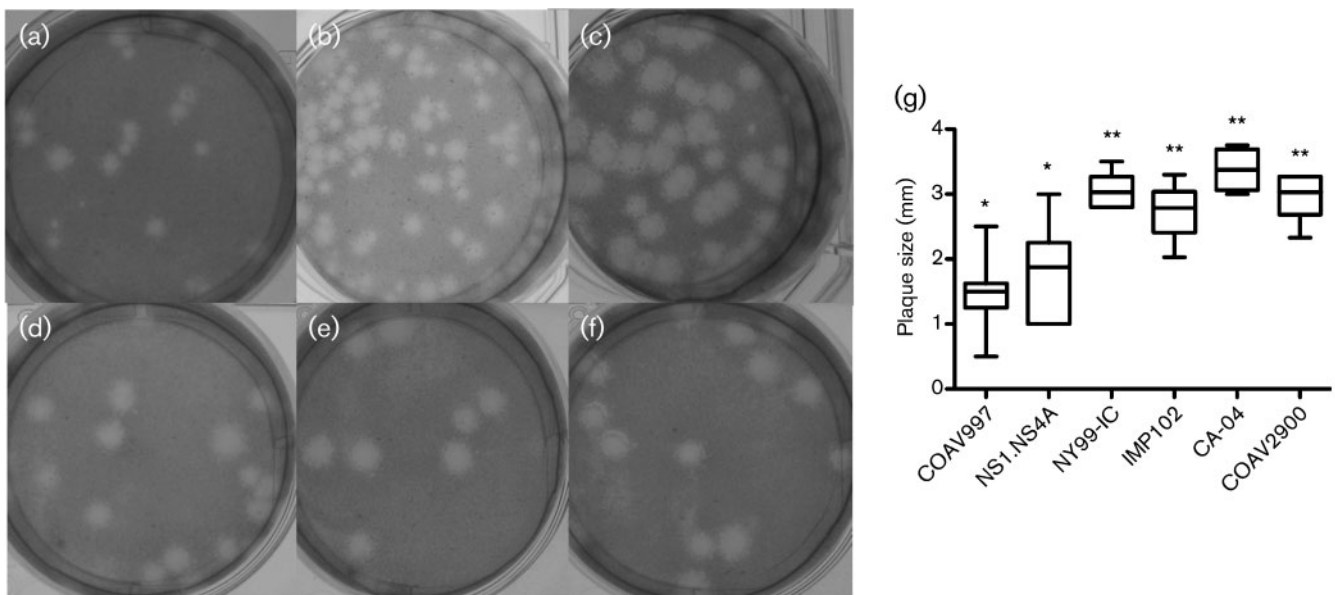


Fig. 5. Plaque phenotypes in Vero cells at 41 °C on day 3 p.i. for comparison of the California strains, NY99-IC and NS1.NS4A mutant: (a) COAV997, (b) NS1.NS4A, (c) NY99-IC, (d) IMP102, (e) CA-04 and (f) COAV2900. (g) Box plot of plaque sizes (mm) with the median value represented as the bar within the box. The 25% quartiles are represented by the edges of the box, and the error bars span from the highest to the lowest measurements. Datasets labelled with the same number of asterisks (*) were not significantly different from one another as determined by Tukey's multiple comparisons test ($P < 0.05$).

NS1-K110N and 3'UTR mutants were not statistically different from the NY99-IC strain at 44 °C, excluding the likelihood of these individual mutations contributing to the observed *ts* phenotype of COAV997. The double mutant containing both the E-V159A and NS1-K110N mutations similarly failed to demonstrate a significantly debilitated *ts* phenotype at 44 °C. However, the triple mutant, E.NS1.3'UTR, did exhibit a significant decrease in overall mean titre at 44 °C, as compared with NY99-IC, thus suggesting a cumulative *ts* effect. It is of interest that the *ts* phenotype was not observed without the addition of the 3'UTR mutant. Davis *et al.* examined *ts* WNV strains isolated in Texas in 2003 and found that their observed *ts* phenotype required either three 3' UTR mutations, which included 3'UTR-a/g10851g, or several non-structural amino acid substitutions in concert with the 3' UTR mutations (Davis *et al.*, 2004, 2007). In the current study, there were no observed phenotypic differences between NY99-IC and E-V159A mutants, E-V159A being the genetic marker for the WN02 genotype; comparisons should be made *in vivo* to further understand the role of this particular non-synonymous mutation, especially in combination with mutants NS1-K110N and 3'UTR-c10772t.

The mean overall titre of the WNV mutant containing the NS4A-F92L individual point mutant, which is the only predicted amino acid variation between COAV997 and IMP102, was not statistically different from NY99-IC, and this served as experimental evidence that the highly *ts* phenotype could not be attributed to this single mutation. However, a recombinant WNV containing both NS1-K110N and NS4A-F92L mutations did demonstrate the complete COAV997 *ts* phenotype at 44 °C, indicating the combined role of NS1-K110N and NS4A-F92L in serving as the genetic determinants mediating this phenotype. This genetic synergism is not completely unexpected as previous *trans*-complementation studies have demonstrated a direct interaction between NS1 and NS4A proteins for the production of a viable replication complex (Lindenbach & Rice, 1999).

The NS1 protein plays a role in flavivirus RNA replication (Brinton, 2002; Lindenbach & Rice, 1999; MacKenzie *et al.*, 1996; Muylaert *et al.*, 1997), and a study utilizing conditional NS1 yellow fever virus mutants identified a highly *ts* virus that was deficient in accumulation of both positive- and negative-strand RNA at the non-permissive temperature (Muylaert *et al.*, 1997). The function of the flaviviral NS4A transmembrane protein is not fully understood, but this small, hydrophobic protein (Brinton, 2002) is known to associate with membranes and has been associated with viral replication complexes (Miller *et al.*, 2007) and evasion of the innate immune response of hosts (Lin *et al.*, 2008). Research with related flaviviruses supports the proposition that the NS4A protein may be involved in mediating host innate immune responses by acting as an interferon antagonist and suppressing the phosphorylation of STAT during infection (Lin *et al.*, 2008). The NS4A protein may also act as an NS3 cofactor

involved in sustaining helicase activity during periods of ATP depletion (Shiryaev *et al.*, 2009). A direct genetic interaction between NS1 and NS4A, potentially critical for RNA replication, was proposed based on the identification of an NS4A amino acid substitution in a yellow fever virus NS1 deletion mutant that allowed for successful complementation with dengue virus NS1 provided in *trans* (Lindenbach & Rice, 1999). The temperature sensitivity observed in COAV997 at 44 °C is potentially because such an interaction between NS1-K110N and NS4A-F92L results in instability of the RNA replication complex at elevated temperatures.

Full-length sequences from additional California strains revealed that two ancillary 2003 mosquito-pool isolates (IMP116 and IMP1075; Table 2) contained the same NS1-K110N and NS4A-F92L mutations that were identified in COAV997. This observation, in a limited number of sequences of isolates made following WNV introduction to California, could indicate the prevalence of these two mutations in introduced WNV strains circulating in the Imperial Valley or as being a possible result of a founder effect as the virus was initially introduced into the desert biome, probably from Arizona (Deardorff *et al.*, 2006). An additional California strain (COAV689), containing the NS1-K110N and NS4A-F92L mutations, was identified from the 2004 transmission season, indicating that viruses containing these two mutations persisted overwinter or were reintroduced the following spring. COAV689 contained additional non-synonymous mutations in the NS4A (NS4A-V135M) and the 3' UTR (3'UTR-t11009c) (Table 2). The NS4A-V135M mutation was also identified in strain IMP1075 from 2003 (Table 2). This additional NS4A mutation may have played a role in stabilizing any *in vivo* phenotypic effect resulting from the NS1-K110N and NS4A-F92L mutations; however, the validity of this assertion would require additional investigation.

The CA-04 strain was isolated in Northern California where the NS1-K110N and NS4A-F92L mutations have not been identified in any published sequences. The COAV2900 strain isolated in the Coachella Valley during 2005 does not contain the NS1-K110N and NS4A-F92L mutations; however, this strain has extensive non-synonymous and non-coding mutations compared with NY99, as well as sequence commonalities with isolates from Colorado and Arizona from previous transmission seasons, thus suggesting a possible common progenitor for these strains. The NS4A-A85T amino acid substitution (present in the COAV2900 virus described here) was also found in strains from Colorado in 2003 (CO2003-2) and Arizona in 2004 (AZ2004) (Davis *et al.*, 2005). AZ2004 has an additional amino acid substitution in common with COAV2900 at NS5-K314R (Davis *et al.*, 2005).

Previous studies reported WNV strains exhibiting *ts* phenotypes in avian cells at extreme temperatures that were associated with debilitated viral titres *in vivo* in either avian or mosquito hosts (Jia *et al.*, 2007; Kinney *et al.*,

2006). Characterization of a small-plaque variant of a New York strain WNV isolated in 2000 that contained non-synonymous mutations in the membrane glycoprotein (prM) and NS2A proteins similarly showed no significant differences when assessed at different temperatures (26, 30 and 34 °C) in C6/36; however, the small plaque variant exhibited significantly lower titres in *Cx. pipiens* when maintained at 15, 28 and 34 °C (Jia *et al.*, 2007). In PDE cells (synonymous with DEF cells) maintained at various temperatures, the small-plaque variant did not produce detectable virus at the high temperature (42.5 °C), as compared with the large-plaque variant (Jia *et al.*, 2007). Kinney *et al.* previously reported that a Kenyan WNV strain with reduced avian virulence potential was also highly *ts* in DEF at 44 °C, a temperature observed in WNV-infected viraemic American crows (Kinney *et al.*, 2006). Interestingly, although only 11 non-synonymous mutations were observed between the Kenyan and NY99 viruses, there was both an amino acid change in NS1 as well as an NS4A-A85V substitution, which is similar to the COAV2900 NS4A-A85T mutation, and only 7 aa upstream of the critical NS4A-92 residue identified as being associated with retarded high temperature replication of the COAV997 strain. The highly *ts* phenotype observed at 44 °C with the COAV997 strain and NS1.NS4A mutant may be associated with a similar low-level avian virulence phenotype or decreased replication in mosquitoes. Investigation of COAV997 and the NS1.NS4A mutant *in vivo* is warranted in order to further elucidate the effect of naturally occurring WNV *ts* strains on replication in mosquito vectors as well as in avian hosts. Unlike mosquito studies that have demonstrated increased dissemination of the WN02 genotype at elevated temperatures (Ebel *et al.*, 2004; Kilpatrick *et al.*, 2008), the results presented herein have failed to identify a temperature-versus-strain interaction for *in vitro* C6/36 growth. One possible factor that could dictate this difference is the lack of a functional RNAi system in C6/36 cells (Brackney *et al.*, 2010). This fact, coupled with a potential association of temperature in mediating RNAi responses (Szittyá *et al.*, 2003), could indicate that temperature and RNA interference pathways might be coupled and that differential antagonistic capacities of WNV strains could be critical for some of the observed *in vivo* differences that are mediated at elevated temperatures. Similar mosquito competence studies will need to be performed to assess this theory further. However, results from this study have allowed the independent assessment of temperature as a direct factor modulating growth of different strains of WNV. Interestingly, corvids are rare in south-eastern California and the primary maintenance and amplification hosts, house finches and house sparrows (Reisen *et al.*, 2008), did not produce elevated temperatures, even following infection with NY99 virus (G. Worwa, unpublished data). The proliferation of viruses carrying the *ts* mutations, NS1-K110N and NS4A-F92L, could have been because of the fact that circulation in areas essentially devoid of corvids removed a selective force present in alternative landscapes

where other passeriforms serve as important amplifying hosts. As previously mentioned, WNV isolates from Northern California, with robust corvid populations, have not been observed to express either *ts* mutation identified in this study.

METHODS

Cells and virus strains. DEF (ATCC accession no. CCL-141) and *Ae. albopictus*-derived mosquito cell (C6/36) cultures were used for temperature-dependent assays. Viral titres were determined with African green monkey kidney cells (Vero cells; ATCC no. CCL-81). Baby hamster kidney cells (BHK-21, clone 13; ATCC no. CCL-10) were utilized during transfection protocols for rescuing infectious-clone-derived viruses. Media used to maintain cell lines: Eagle's minimum essential medium (ATCC) for DEF cells, Schneider's *Drosophila* medium (Gibco) for C6/36 cells, Dulbecco's minimal essential medium with low glucose (Gibco) for Vero cells and minimal essential medium (Gibco) for BHK cells. All media were routinely supplemented with 10% FBS and penicillin/streptomycin (100 U ml⁻¹; Gibco).

An infectious clone-derived virus based on NY99 strain 382-99 was generated as previously described (Beasley *et al.*, 2005; Kinney *et al.*, 2006). The COAV997 and CA-03 IMPR 102 (IMP102) isolates were made from *Cx. tarsalis* mosquito pools collected in 2003 from the Imperial Valley. CA-04 04-7168 (CA-04) was isolated from a yellow-billed magpie (*P. nuttalli*) that died in Sacramento County in 2004. The COAV2900 isolate was generated from a *Cx. tarsalis* mosquito pool made in 2005 having been collected from the Coachella Valley. All California isolates were made in Vero cell culture (Table 1).

Full-length sequencing of natural isolates. Viral RNA was obtained from isolates after passage in Vero cells with a QIAamp Viral RNA Mini kit (Qiagen), following the manufacturer's protocols. Primers were designed based on North American genomic sequences. SuperScript II Reverse Transcriptase (Invitrogen) and *PfuUltra* (Stratagene) were used to generate cDNA and PCR amplicons, respectively. To amplify the 5' and 3' ends of the genome, 5' RACE and 3' RACE Systems (Invitrogen) were used. The poly(A) tail necessary for the 3' RACE amplification was engineered with a Poly-A Plus kit (Epicentre), following the manufacturer's protocol. A Qiaquick gel extraction kit (Qiagen) was used to clean RT-PCR products, and sequencing was performed on an ABI capillary sequencer.

Construction and rescue of clone-derived infectious viruses. A two-plasmid system based on NY99 (Kinney *et al.*, 2006) was utilized to engineer an infectious-clone-derived version of the COAV997 natural isolate (COAV997-IC) containing E-V159A, NS1-K110N, NS4A-F92L and 3'UTR-c10772t mutations. Additionally, mutant viruses were engineered to insert individual or combinations of non-synonymous COAV997 point mutants into the NY99 virus backbone. The following clone-derived viruses containing individual or combinations of COAV997 mutations were engineered: (i) E-V159A, (ii) NS1-K110N, (iii) NS4A-F92L, (iv) 3'UTR-c10772t, (v) E-V159A and NS1-K110N, (vi) NS1-K110N and NS4A-F92L, and (vii) E-V159A, NS1-K110N and 3'UTR-c10772t. Of note, the NY99-IC was engineered with 3'UTR-a/g10851g and therefore all viruses derived from this strain contain this nucleotide configuration. Briefly, NY99-plasmid was mutagenized by using a QuikChange site-directed mutagenesis kit (Stratagene), and the presence of the desired mutations was confirmed by direct sequencing. Specific mutagenesis primers were designed to insert each individual point mutation on either the 5' (5'UTR-NS1) or 3' (NS1-3'UTR) plasmids by using wrap-around PCR amplification. Mutants containing more than one point mutant were engineered by using multiple rounds of mutagenesis, with altered genome sequences

being confirmed between rounds. The 5' and 3' plasmids were digested and ligated to serve as template for RNA transcription. The viral RNA generated was transfected into BHK-21 cells (Kinney *et al.*, 2006). Cells were observed daily for cytopathic effect, as compared with a negative control. Virus was harvested 3–5 days p.i. when 2+ cytopathogenicity was observed. Harvested supernatants were titrated on Vero cells and full-length sequencing performed from amplicons generated to assess the sequence identity of all derived viruses.

Temperature-dependent replication. C6/36 and DEF cultures were grown in 25 cm² flasks each inoculated in triplicate at an m.o.i. of 0.1. Initial inocula were later back-titrated to confirm m.o.i. Viruses were adsorbed for 90 min at 28 °C (C6/36) or 37 °C (DEF) in a 5% CO₂ atmosphere. After the adsorption period, the monolayer was washed twice with PBS (pH 7.4), 6 ml of maintenance medium containing 10% FBS was added and the cultures were incubated at the selected temperatures. Inoculated cultures of C6/36 were incubated at 22, 28 or 34 °C without CO₂, while DEF cultures were incubated at 37, 41 and 44 °C in a 5% CO₂ atmosphere. Samples were collected at 24 h intervals, diluted 1:10 in medium supplemented with 20% FBS, and stored at –80 °C until being assayed for infectious titre.

Viral titration. Infectious viral titres were quantified by serial tenfold dilutions of virus samples on Vero cells as previously described (Brault *et al.*, 2004). Briefly, virus dilutions were allowed to adsorb for 1 h at 37 °C before the addition of a nutrient overlay containing 0.5% agarose. A second overlay containing 0.005% neutral red was added at day 2 p.i. P.f.u. were enumerated at day 3 p.i. and used to calculate the titre (log₁₀ p.f.u. ml⁻¹) of the sample. In order to document plaque morphology and size at the experimental temperatures, the viral titration protocol was performed as described above, except that after application of the overlay the cells were maintained at 37 °C or 41 °C (Vero cells did not tolerate temperatures >41 °C). Digital images and measurements were made on day 3 p.i. Vero cells were used as a neutral cell line for plaque morphology comparisons owing, in part, to poor plaque resolution in DEF cells, especially at 44 °C where non-virus-induced cytopathic effect has been observed. Additionally, plaque morphology measurements were taken at 41 °C in order to minimize the effects of agar overlay viscosity that have been observed at 44 °C (unpublished results).

Statistical analysis. A repeated measures ANOVA (Hintze, 1998) was utilized to determine statistical significance between viral strains and temperatures, with days p.i. as the within repeated flask factor. If a significant virus strain versus temperature interaction was observed, then the repeated measures ANOVA was repeated with data specific for each temperature. *Post-hoc* analysis was performed by using the Tukey–Kramer test for all pairwise multiple comparisons between the means with $\alpha=0.05$.

Plaque measurements were analysed by using the Graph Pad Prism (version 5) software. A one-way ANOVA was used to determine whether there was statistically significant variation in the plaque size of the viruses. If a significant difference was observed, then a Tukey's multiple comparisons test was used as a *post-hoc* analysis with $P<0.05$.

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